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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

Attorney's Docket No. 2328-124

U.S. Application No. (if known, see 37 CFR 1.5) 09/980585

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FI00/00507	07 June 2000	07 June 1999

TITLE OF INVENTION: A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS

APPLICANT(S) FOR DO/EO/US: Esa-Matti LILIUS and Marko VIRTA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

ITEMS 11. TO 20. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A **FIRST** preliminary amendment.
14. [ ] A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. [X] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: Sequence Listing (10 pages); Five (5) sheets of drawings; Applicant(s) Data Sheet; Statement Pursuant to 37 CFR §1.821(f); and Statement Pursuant to 37 CFR §1.125 with marked-up copy of substitute specification.

JC13 Rec'd PCT/PTO 0 4 DEC 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.50) 09/980585	INTERNATIONAL APPLICATION NO. PCT/FI00/00507	ATTORNEY DOCKET NO. 2328-124

	CALCULATIONS	PTO USE ONLY
21. [X] The following fees are submitted: **Basic National Fee (37 CFR 1.492)(a)(1)-(5):** Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report Not Prepared by EPO or JPO.......... $ 1,040.00 International preliminary examination fee (37 CFR 1.482) not paid t o USPTO but International Search Report has been prepared by the EPO or JPO.......... $ 890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.......... $ 740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4).......... $ 710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).......... $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1,040.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	10 -20 =	0	X $18.00	$00.00	
Independent Claims	01 - 3 =	0	X $84.00	$00.00	
Multiple dependent claim(s) (if applicable)			+ $280.00	$00.00	
TOTAL OF ABOVE CALCULATIONS =				$1,040.00	
[X] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$520.00	
SUBTOTAL =				$520.00	
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$520.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$520.00	
				Amount to be refunded	$
				charged	$

a. [X] A check in the amount of $520.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. 02-2135 in the amount of $________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2135. A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Customer No. 6449

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Signature

Jeffrey L. Ihnen
Name

28,957
Registration Number

09980585 .120401

09/980585
JC13 Rec'd PCT/PTO 04 DEC 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	*Application Number*	New Application
	Filing Date	Herewith
	First Named Inventor	Esa-Matti LILIUS
	Group Art Unit	Unassigned
	Examiner Name	Unassigned
	Attorney Docket No.	2328-124
Title of the Invention:	**A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS**	

STATEMENT PURSUANT TO 37 CFR 1.125(b)

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In the matter of the above-identified nonprovisional application, filed concurrently herewith, Applicants are submitting a substitute copy of the specification. The substitute specifcation contains no new matter. A marked-up version of the substitute specification, in which additions are indicated by underlining and deletions are indicated by brackets, is attached.

RESPECTFULLY SUBMITTED,					
NAME AND REG. NUMBER	Jeffrey L. Ihnen, Reg. No. 28,957				
SIGNATURE	Jeffrey L. Ihnen		DATE	04 December 2001	
Address	ROTHWELL, FIGG, ERNST & MANBECK, pc Suite 701-East, 555 13th Street, N.W.				
City	Washington	*State*	D.C.	*Zip Code*	20004
Country	U.S.A.	*Telephone*	202-783-6040	*Fax*	202-783-6031

<table>
<tr><td rowspan="6">IN THE
UNITED STATES
PATENT AND TRADEMARK
OFFICE</td><td>Application Number</td><td>09/980,585</td></tr>
<tr><td>Filing Date</td><td>4 December 2001</td></tr>
<tr><td>First Named Inventor</td><td>Esa-Matti LILIUS</td></tr>
<tr><td>Group Art Unit</td><td>Unassigned</td></tr>
<tr><td>Examiner Name</td><td>Unassigned</td></tr>
<tr><td>Attorney Docket No.</td><td>2328-124</td></tr>
<tr><td>Title of the Invention:</td><td colspan="2">A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS</td></tr>
</table>

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In response to the Notice of Missing Requirements, response copy attached, please amend the above-identified application as follows.

<u>IN THE SEQUENCE LISTING:</u>

Please replace the originally filed Sequence Listing with the substitute Sequence Listing attached hereto.

REMARKS

The Sequence Listing has been revised in view of the inability of the programs to correctly number and accept a nucleic acid sequence which contains more than one coding sequence. Consequently, each sequence originally identified as "CDS" in SEQ ID NO:1 of the original Sequence Listing have been relabeled as "gene" to prevent any errors with the Checker software. The proteins encoded by these "genes" are set forth in SEQ ID Nos:2-4 as in the original Sequence Listing. The substitute Sequence Listing contains no new matter and its entry is requested.

A computer readable copy of the Sequence Listing is submitted concurrently herewith.

RESPECTFULLY SUBMITTED,			
NAME AND REG. NUMBER	Jeffrey L. Ihnen, Reg. No. 28,957		
SIGNATURE		*DATE*	04 March 2002

Address	ROTHWELL, FIGG, ERNST & MANBECK, pc 1425 K Street, N.W., Suite 800				
City	Washington	*State*	D.C.	*Zip Code*	20005
Country	U.S.A.	*Telephone*	202-783-6040	*Fax*	202-783-6031

Attachment: substitute Sequence Listing

09/980585
JC13 Rec'd PCT/PTO 04 DEC 2001

TITLE OF THE INVENTION

A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application is a national stage filing under 35 U.S.C. §371 of PCT/FI00/00507 filed on 7 June 2000 and claims priority under 35 U.S.C. §119 to Finland patent application No. 991296 filed on 7 June 1999.

[0002] This invention relates to a method to enable the assessment of growth and death of a micro-organism within a chosen time period in an environment of interest.

BACKGROUND OF THE INVENTION

[0003] When studying growth and death of a micro-organism under the influence of specific environments, e.g. production and storage environments that e.g. could or could not be refrigerated, or involving chemicals or matrixes, e.g. antibiotics, microbial toxins, heavy metals and serum complement, microbial cultures are most often incubated for hours or days. In these circumstances death and growth occur simultaneously. If additionally some of the cells lyse, e.g. when analysing the serum complement, it is difficult to know to what one should compare the amount of living cells at the end of the experiment. Convenient methods to determine the number of living cells, e.g. by measuring luciferase bioluminescence, are known but if no more information is available it is impossible to assess to what extent growth or/and death of the micro-organisms takes or has taken place.

[0004] Growth rates and death rates of micro-organisms in specific environments are of interest in many areas. Death rates and growth rates of micro-organisms and especially harmful and/or pathogenic micro-organisms are of importance in risk assessments of products of the pharmaceutical industry and products for human consumption with regard to their production, storage and distribution to the consumers. Knowledge of death and growth rates of micro-organisms are of particular importance in specific applications such as in the development of antibiotics, disinfectants and bactericidal products or monitoring of sterilisation, disinfection and cleaning processes.

[0005] Reporter genes coding for luminescent or/and fluorescent products have been introduced to micro-organisms to enable the assessment of the quantity or survival of living

micro-organisms (WO 96/23898, WO 98/14605, WO 98/30715, WO 98/36081, US 5,824,468). Even simultaneous use of luminescent and fluorescent markers has been used (Fratamico et al., Journal of Food Protection, Vol 50 No 10, 1997, 1167–1173). Luminescent and fluorescent markers have, however, only been used as markers for survival of micro-organisms and the use of two different markers within one micro-organism enabling the differentiation between growth and death rates has not been reported.

OBJECT AND SUMMARY OF THE INVENTION

[0006] The object of the present invention is to provide a method to enable the assessment of the growth and death of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism at least two reporter genes. The method is characterised in that

a) said reporter genes code for luminescent and/or fluorescent products and within said time period and environment at least two said products of the following are produced:

   i) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period,

   ii) a product present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period and

   iii) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that have died within said chosen time period,

   and said products can be measured through their luminescence and/or fluorescence;

b) said micro-organism is incubated within the environment of interest and said luminescence and/or fluorescence is detected after said chosen time period, and

c) the growth and death rate of the said micro-organism is assessed based on at least two of the following:

i) the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period,

ii) the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period, and

iii) the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] Figure 1 shows plasmid pGFP+luc* including genes for both GFP and firefly luciferase.

[0008] Figure 2 shows fluorescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0009] Figure 3 shows luminescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0010] Figure 4 shows the amount of living cells, i.e. colony forming units, according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0011] Figure 5 shows the percentage of living cells according to live/dead staining and flow cytometric analysis during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0012] Figure 6 shows fluorescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

[0013] Figure 7 shows luminescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

[0014] Figure 8 shows the percentage of living cells according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The method according to the present invention can be used to assess the growth and death rate of a micro-organism within a chosen time period in any particular environment of interest. The method is applicable if two different marker genes can be introduced to the micro-organism that code for luminescent and/or fluorescent products, and the products of these fulfil at least two of the following three criteria:

a) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism alive within said chosen time period ;

b) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism that are or have been alive within said chosen time period, and

c) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism that have died within said chosen time period.

[0016] In the present application the concept "micro-organism" means any micro-organism into which marker genes can be introduced so, that they will function according to the invention. "Micro-organism" can therefore stand for bacteria, yeast or fungi.

[0017] The concept of "introducing a marker gene into a micro-organism" means any method by which a marker gene can be made to function within the micro-organism according to the invention. One way of introducing marker genes into micro-organism is by constructing a recombinant strain. This can be done by transforming a strain with a plasmid including the marker genes. An alternative way to introduce reporter genes to bacteria is to utilise transposable elements. In this technique, reporter genes are inserted between insertion sequences in a delivery plasmid. The plasmid is then introduced to a cell by e.g. conjugation of transformation, and once inside the cell, genes surrounded by the insertion sequences are integrated into bacterial chromosome. Integration is stable, i.e. there is no need for a selectable marker such as antibiotic resistance.

[0018] Assessment of the growth rate and death rate of a micro-organism can be of interest in many specific environments. Within pharmaceutical research the effect of different drugs and candidates for drugs, e.g. antibiotics, on the survival of pathogenic, but also the

beneficial micro-organisms of the gut, is of interest. Thus the ultimate interest is in the behaviour of these micro-organisms in a physiological environment affected by drugs.

[0019] Another vast area where the possibility of assessing growth and death rate of specific micro-organisms is of interest is that of production, processing, storage and distribution of all products for human consumption. In this area the behaviour of pathogenic or potentially harmful micro-organisms in the different environments of the life cycle of these products is of special interest and involves many different aspects such as the influence of temperature, humidity or light and the possible use of preservatives etc.

[0020] Additionally growth and death rates of micro-organisms can be of interest for environmental evaluations e.g. when evaluating the effect of emissions into the environment.

[0021] Luminescent or fluorescent products coded by reporter genes in different embodiments of this invention can vary as long as their proportion to either the total amount of cells alive, to cells that are or have been alive, or to cells that have died is essentially known. Growth and death rate can be assessed if two of the following: cells alive, cells that are or have been alive, or cells that have died can be determined. Thus luminescence and/or fluorescence measured can be e.g. of a product which is expressed e.g. constitutively or triggered by a specific phase (e.g. replication or death) of the lifecycle of each cell, is stable or labile or which luminescence or fluorescence is dependant on a factor that relates e.g. to a specific phase of the lifecycle of each cell. Depending on the individual characteristics of said product—how produced, stable or labile, possible dependence of its luminescence or fluorescence of said factors etc.— the measured luminescence or fluorescence can be in proportion to one of the three unknown of which two must be known to be able to assess the growth rate and death rate of said cells.

[0022] According to one specific embodiment of the invention assessment of the growth and death rate of an *Esherichia coli* strain under the influence of different chemicals or matrixes was enabled by constructing a recombinant strain, which expresses both luciferase and GFP. Altogether the effect of a number of different chemicals and matrixes, such as $CdCl_2$, ethanol, the antibiotics chloramphenicol, rifampicin, and tetracyclin, as well as serum complement on said recombinant *E. coli* strain was tested and found applicable.

[0023] The invention will be described in more detail by the following study in which the growth rate and death rate of a recombinant *Esherichia coli* strain, which expresses both luciferase and GFP, is assessed under the influence of ethanol or serum complement.

Summary of the study

[0024] Genes for luciferase and green fluorescent protein have recently raised interest as reporter genes. Luciferase is an enzyme that produces luminescence in the presence of substrate luciferin, molecular oxygen and ATP. Green fluorescent protein (GFP), produces green fluorescence when excited with light. Many mutated forms of GFP have been introduced: some have different excitation and emission wavelengths from the wild type and some mutants form more stable proteins at higher temperatures.

[0025] We constructed a recombinant strain of *E. coli*, which expresses both luciferase and GFP. In our construction we used a mutant of GFP, which is more stable at temperatures over +30 °C and it matures quicker than the wild type. Luciferase was from North American firefly, *Photinus pyralis*.

[0026] The *E. coli* strain MC1061 was transformed with a plasmid including genes for both GFP and firefly luciferase. Figure 1 describes the plasmid in general. The sequence of the plasmid is disclosed in the sequence listing. Essential codings of the sequence are as follows:

lac promoter	95–199
GFP	289–1008
firefly luciferase	1044–2696
β-lactamase	3251–4111

[0027] In our construct, see Figure 1, the luciferase gene is situated next to the GFP gene and both genes are transcribed in the same direction. The transcription is started at the lac promoter in front of GFP. The lac promoter is constitutively active, because the MC1061 cells lack its repressor. The plasmid also has a gene for ampicillin resistance (β-lactamase).

[0028] The transformed *E. coli* strain was propagated under the influence of different concentrations of ethanol or serum complement.

Methods

Growth conditions

[0029] One colony from a pure culture plate was inoculated in 5 ml of LB-medium with ampicillin (100 μg/ml) and grown at +37 °C in a shaker, 250 rpm, for about three hours. After that, the number of cells per millilitre was determined with flow cytometry by using fluorescent spherical latex particles as a reference. One million cells were then removed to an erlenmeyer

with 50 ml of LB medium and ampicillin. The culture was grown over night in a shaker, 190 rpm, at room temperature to prevent the culture from growing into the stationary phase during the night. In the morning, the culture was transferred to and grown in a shaker, 330 rpm, until the stationary phase was reached or used after growth at +30 °C for about 1 h to study the influence of ethanol or serum complement as described below.

<u>Influence of chemicals on the propagation of *E. coli*</u>

[0030] The culture obtained as described above was used to study the influence of ethanol or serum complement as follows:

Ethanol

[0031] Ethanol (Aa, Primalco Oy) was diluted into pure water to obtain concentrations of 50, 45, 25, 10, 5, 1 and 0 % of ethanol when 500 µl of said dilution was added to 500 µl of said culture in an eppendorf tube. The mixture was vortexed and incubated for 5 minutes before measuring fluorescence and luminescence. Live cells were again counted by plating and also by live/dead staining. In the live/dead protocol used the stain *cyto 9* stains all cells whereas propidium iodide stains only the dead cells. After staining, cells are passed through a flow cytometer, with which dead and live cells can be differentiated and their proportion determined. (Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.)

Serum complement

[0032] The influence of serum complement on the said recombinant *E. coli* strain was studied using an incubation time of 90 min as described for a different recombinant *E. coli* strain used in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.

<u>Fluorescence and luminescence measurements</u>

[0033] The measurements were done with a combined fluoro- and luminometer, Fluoroscan Ascent FL, provided by Labsystems Ltd. (Helsinki, Finland). Cell growth was simultaneously followed with a flow cytometer.

[0034] For the measurements, 100 µl of bacterial culture was pipetted into the microtiter plate wells. Fluorescence was measured using 485 nm for excitation and 510 nm for emission. Measuring time was 20 ms. After the fluorescence measurement 100 µl of luciferin in 0.1 M citric acid-sodium citrate buffer (pH 5.0) was dispensed into the wells and the plate was shaken

for two minutes (shaking diameter 1 mm, 1 020 rpm), after which luminescence was recorded with a measuring time of 1000 ms.

Plating

[0035] Samples for plating were diluted 10^2 to 10^7 fold with 150 mM NaCl and plated onto L agar plates (L broth containing 1.6 % agar). Colonies were counted after overnight incubation at 37 °C.

Live/dead staining and Flow cytometric analysis

[0036] Bacteria from 1 000 μl of cell culture were used for live/dead staining and flow cytometric analysis using a LIVE/DEAD BacLight bacterial viability kit (catalogue no. L-7005) for microscopy and quantitative analysis obtained from Molecular Probes Europe (Leiden, The Netherlands) and Fluoresbrite beads (diameter, 1.8 μm) obtained from Polysciences Inc. (Warrington, Pa.) as described in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.

Results

[0037] When the cultures were transferred to +30 °C, the cells grew logarithmically for 1-4 hours depending on the initial cell concentration. Luminescence and fluorescence rose logarithmically and were essentially constant per cell. Thus cell number could be assessed based on luminescence or fluorescence.

[0038] When ethanol was added in different concentrations to the growth medium (see Figures 4 and 5) death was, after a very short incubation period of 5 min, more or less unsignificant at ethanol concentrations below 5 % and became more significant with increasing ethanol concentration reaching very pronounced significance at ethanol concentrations above 10 %. Correspondingly fluorescence (Figure 2) was essentially constant whatever the ethanol concentration in spite of dramatically decreasing corresponding live cell count according to plate count (Figure 4) and percentage of live cells according to the live/dead staining (Figure 5) whereas luminescence (Figure 3) dropped dramatically essentially corresponding to the dramatic drop in plate count (Figure 4) and the percentage of live cells (Figure 5) with increased ethanol concentration.

[0039] The effect of serum complement on the growth and death of *E. coli* is shown in Figures 6 to 8. Fluorescence (Figure 6) and luminescence (Figure 7) are shown before (squares) and after (circles) incubation for 90 minutes with serum complement. Fluorescence (Figure 6) is

slightly increased, during incubation regardless of the concentration of serum, whereas luminescence (Figure 7) decreases during incubation with increasing serum concentration. The decrease of luminescence during incubation with increasing concentrations of serum correlates clearly with the percentage of cells alive after incubation (Figure 8).

CLAIMS

1. A method to enable the assessment of the growth rate and death rate of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism at least two reporter genes, which method is characterised in that

a) said reporter genes code for luminescent and/or fluorescent products and within said time period and environment at least two said products of the following are produced:

i) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period,

ii) a product present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period, and

iii) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that have died within said chosen time period,

and said products can be measured through their luminescence and/or fluorescence;

b) said micro-organism is incubated within the environment of interest and said luminescence and/or fluorescence is detected after said chosen time period, and

c) the growth and death rate of the said micro-organism is assessed based on at least two of the following:

i) the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period,

ii) the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period, and

iii) the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

2. The method according to claim 1 characterised in that said micro-organism is a gram negative bacteria, e.g. *Escherichia coli*.

3. The method according to claim 1 [or 2] characterised in that

a) one reporter gene coding for a luminescent product is luciferase, which is used for the determination of amount of cells alive at any moment within said chosen time period, and

b) another reporter gene coding for a fluorescent product is green fluorescent protein (GFP), which is used for the determination of total amount of cells of said micro-organism that are or have been alive within said chosen time period.

4. The method according to claim 1 [or 2] characterised in that said reporter genes are introduced into said micro-organism in a plasmid.

5. The [A] method according to [the methods of] claim 3 [or 4] characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

6. The method according to claim 2 characterised in that

a) one reporter gene coding for a luminescent product is luciferase, which is used for the determination of amount of cells alive at any moment within said chosen time period, and

b) another reporter gene coding for a fluorescent product is green fluorescent protein (GFP), which is used for the determination of total amount of cells of said micro-organism that are or have been alive within said chosen time period.

7. The method according to claim 2 characterised in that said reporter genes are introduced into said micro-organism in a plasmid.

8. The method according to claim 4 characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

9. The method according to claim 6 characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

10. The method according to claim 7 characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

ABSTRACT

[0040] A method to enable the assessment of the growth rate and death rate of a micro-organism within a chosen time period in an environment of interest. The method is characterised in that

a) two reporter genes are introduced to said micro-organism wherein, the reporter genes used code for luminescent and/or fluorescent products and at least two of the following products: an essentially stable product produced in an essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period; a product present in an essentially known proportion to the amount of cells alive at any moment within said chosen time period; and an essentially stable product produced in an essentially known proportion to the total amount of cells of the said micro-organism that have died within said chosen time period, and said products can be measured through their luminescence and/or fluorescence;

b) the said micro-organism is incubated and said luminescence and/or fluorescence is detected after said chosen time periods, and

c) the growth and death rate of the said micro-organism is assessed based on at least two of the following: the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period; the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period; and the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

JC13 Rec'd PCT/PTO 04 DEC 2001 09/980585

5/prts

TITLE OF THE INVENTION

A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application is a national stage filing under 35 U.S.C. §371 of PCT/FI00/00507 filed on 7 June 2000 and claims priority under 35 U.S.C. §119 to Finland patent application No. 991296 filed on 7 June 1999.

[0002] This invention relates to a method to enable the assessment of growth and death of a micro-organism within a chosen time period in an environment of interest.

BACKGROUND OF THE INVENTION

[0003] When studying growth and death of a micro-organism under the influence of specific environments, e.g. production and storage environments that e.g. could or could not be refrigerated, or involving chemicals or matrixes, e.g. antibiotics, microbial toxins, heavy metals and serum complement, microbial cultures are most often incubated for hours or days. In these circumstances death and growth occur simultaneously. If additionally some of the cells lyse, e.g. when analysing the serum complement, it is difficult to know to what one should compare the amount of living cells at the end of the experiment. Convenient methods to determine the number of living cells, e.g. by measuring luciferase bioluminescence, are known but if no more information is available it is impossible to assess to what extent growth or/and death of the micro-organisms takes or has taken place.

[0004] Growth rates and death rates of micro-organisms in specific environments are of interest in many areas. Death rates and growth rates of micro-organisms and especially harmful and/or pathogenic micro-organisms are of importance in risk assessments of products of the pharmaceutical industry and products for human consumption with regard to their production, storage and distribution to the consumers. Knowledge of death and growth rates of micro-organisms are of particular importance in specific applications such as in the development of antibiotics, disinfectants and bactericidal products or monitoring of sterilisation, disinfection and cleaning processes.

[0005] Reporter genes coding for luminescent or/and fluorescent products have been introduced to micro-organisms to enable the assessment of the quantity or survival of living

micro-organisms (WO 96/23898, WO 98/14605, WO 98/30715, WO 98/36081, US 5,824,468). Even simultaneous use of luminescent and fluorescent markers has been used (Fratamico et al., Journal of Food Protection, Vol 50 No 10, 1997, 1167–1173). Luminescent and fluorescent markers have, however, only been used as markers for survival of micro-organisms and the use of two different markers within one micro-organism enabling the differentiation between growth and death rates has not been reported.

OBJECT AND SUMMARY OF THE INVENTION

[0006] The object of the present invention is to provide a method to enable the assessment of the growth and death of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism at least two reporter genes. The method is characterised in that

a) said reporter genes code for luminescent and/or fluorescent products and within said time period and environment at least two said products of the following are produced:
    i) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period,
    ii) a product present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period and
    iii) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that have died within said chosen time period,

    and said products can be measured through their luminescence and/or fluorescence;
b) said micro-organism is incubated within the environment of interest and said luminescence and/or fluorescence is detected after said chosen time period, and
c) the growth and death rate of the said micro-organism is assessed based on at least two of the following:

i) the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period,

ii) the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period, and

iii) the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] Figure 1 shows plasmid pGFP+luc* including genes for both GFP and firefly luciferase.

[0008] Figure 2 shows fluorescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0009] Figure 3 shows luminescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0010] Figure 4 shows the amount of living cells, i.e. colony forming units, according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0011] Figure 5 shows the percentage of living cells according to live/dead staining and flow cytometric analysis during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

[0012] Figure 6 shows fluorescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

[0013] Figure 7 shows luminescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

[0014] Figure 8 shows the percentage of living cells according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The method according to the present invention can be used to assess the growth and death rate of a micro-organism within a chosen time period in any particular environment of interest. The method is applicable if two different marker genes can be introduced to the micro-organism that code for luminescent and/or fluorescent products, and the products of these fulfil at least two of the following three criteria:

a) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism alive within said chosen time period ;

b) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism that are or have been alive within said chosen time period, and

c) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism that have died within said chosen time period.

[0016] In the present application the concept "micro-organism" means any micro-organism into which marker genes can be introduced so, that they will function according to the invention. "Micro-organism" can therefore stand for bacteria, yeast or fungi.

[0017] The concept of "introducing a marker gene into a micro-organism" means any method by which a marker gene can be made to function within the micro-organism according to the invention. One way of introducing marker genes into micro-organism is by constructing a recombinant strain. This can be done by transforming a strain with a plasmid including the marker genes. An alternative way to introduce reporter genes to bacteria is to utilise transposable elements. In this technique, reporter genes are inserted between insertion sequences in a delivery plasmid. The plasmid is then introduced to a cell by e.g. conjugation of transformation, and once inside the cell, genes surrounded by the insertion sequences are integrated into bacterial chromosome. Integration is stable, i.e. there is no need for a selectable marker such as antibiotic resistance.

[0018] Assessment of the growth rate and death rate of a micro-organism can be of interest in many specific environments. Within pharmaceutical research the effect of different drugs and candidates for drugs, e.g. antibiotics, on the survival of pathogenic, but also the

beneficial micro-organisms of the gut, is of interest. Thus the ultimate interest is in the behaviour of these micro-organisms in a physiological environment affected by drugs.

[0019] Another vast area where the possibility of assessing growth and death rate of specific micro-organisms is of interest is that of production, processing, storage and distribution of all products for human consumption. In this area the behaviour of pathogenic or potentially harmful micro-organisms in the different environments of the life cycle of these products is of special interest and involves many different aspects such as the influence of temperature, humidity or light and the possible use of preservatives etc.

[0020] Additionally growth and death rates of micro-organisms can be of interest for environmental evaluations e.g. when evaluating the effect of emissions into the environment.

[0021] Luminescent or fluorescent products coded by reporter genes in different embodiments of this invention can vary as long as their proportion to either the total amount of cells alive, to cells that are or have been alive, or to cells that have died is essentially known. Growth and death rate can be assessed if two of the following: cells alive, cells that are or have been alive, or cells that have died can be determined. Thus luminescence and/or fluorescence measured can be e.g. of a product which is expressed e.g. constitutively or triggered by a specific phase (e.g. replication or death) of the lifecycle of each cell, is stable or labile or which luminescence or fluorescence is dependant on a factor that relates e.g. to a specific phase of the lifecycle of each cell. Depending on the individual characteristics of said product—how produced, stable or labile, possible dependence of its luminescence or fluorescence of said factors etc.— the measured luminescence or fluorescence can be in proportion to one of the three unknown of which two must be known to be able to assess the growth rate and death rate of said cells.

[0022] According to one specific embodiment of the invention assessment of the growth and death rate of an *Esherichia coli* strain under the influence of different chemicals or matrixes was enabled by constructing a recombinant strain, which expresses both luciferase and GFP. Altogether the effect of a number of different chemicals and matrixes, such as $CdCl_2$, ethanol, the antibiotics chloramphenicol, rifampicin, and tetracyclin, as well as serum complement on said recombinant *E. coli* strain was tested and found applicable.

[0023] The invention will be described in more detail by the following study in which the growth rate and death rate of a recombinant *Esherichia coli* strain, which expresses both luciferase and GFP, is assessed under the influence of ethanol or serum complement.

Summary of the study

[0024] Genes for luciferase and green fluorescent protein have recently raised interest as reporter genes. Luciferase is an enzyme that produces luminescence in the presence of substrate luciferin, molecular oxygen and ATP. Green fluorescent protein (GFP), produces green fluorescence when excited with light. Many mutated forms of GFP have been introduced: some have different excitation and emission wavelengths from the wild type and some mutants form more stable proteins at higher temperatures.

[0025] We constructed a recombinant strain of *E. coli*, which expresses both luciferase and GFP. In our construction we used a mutant of GFP, which is more stable at temperatures over +30 °C and it matures quicker than the wild type. Luciferase was from North American firefly, *Photinus pyralis*.

[0026] The *E. coli* strain MC1061 was transformed with a plasmid including genes for both GFP and firefly luciferase. Figure 1 describes the plasmid in general. The sequence of the plasmid is disclosed in the sequence listing. Essential codings of the sequence are as follows:

lac promoter	95–199
GFP	289–1008
firefly luciferase	1044–2696
β-lactamase	3251–4111

[0027] In our construct, see Figure 1, the luciferase gene is situated next to the GFP gene and both genes are transcribed in the same direction. The transcription is started at the lac promoter in front of GFP. The lac promoter is constitutively active, because the MC1061 cells lack its repressor. The plasmid also has a gene for ampicillin resistance (β-lactamase).

[0028] The transformed *E. coli* strain was propagated under the influence of different concentrations of ethanol or serum complement.

Methods

<u>Growth conditions</u>

[0029] One colony from a pure culture plate was inoculated in 5 ml of LB-medium with ampicillin (100 μg/ml) and grown at +37 °C in a shaker, 250 rpm, for about three hours. After that, the number of cells per millilitre was determined with flow cytometry by using fluorescent spherical latex particles as a reference. One million cells were then removed to an erlenmeyer

with 50 ml of LB medium and ampicillin. The culture was grown over night in a shaker, 190 rpm, at room temperature to prevent the culture from growing into the stationary phase during the night. In the morning, the culture was transferred to and grown in a shaker, 330 rpm, until the stationary phase was reached or used after growth at +30 °C for about 1 h to study the influence of ethanol or serum complement as described below.

Influence of chemicals on the propagation of *E. coli*

[0030] The culture obtained as described above was used to study the influence of ethanol or serum complement as follows:

Ethanol

[0031] Ethanol (Aa, Primalco Oy) was diluted into pure water to obtain concentrations of 50, 45, 25, 10, 5, 1 and 0 % of ethanol when 500 μl of said dilution was added to 500 μl of said culture in an eppendorf tube. The mixture was vortexed and incubated for 5 minutes before measuring fluorescence and luminescence. Live cells were again counted by plating and also by live/dead staining. In the live/dead protocol used the stain *cyto 9* stains all cells whereas propidium iodide stains only the dead cells. After staining, cells are passed through a flow cytometer, with which dead and live cells can be differentiated and their proportion determined. (Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.)

Serum complement

[0032] The influence of serum complement on the said recombinant *E. coli* strain was studied using an incubation time of 90 min as described for a different recombinant *E. coli* strain used in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.

Fluorescence and luminescence measurements

[0033] The measurements were done with a combined fluoro- and luminometer, Fluoroscan Ascent FL, provided by Labsystems Ltd. (Helsinki, Finland). Cell growth was simultaneously followed with a flow cytometer.

[0034] For the measurements, 100 μl of bacterial culture was pipetted into the microtiter plate wells. Fluorescence was measured using 485 nm for excitation and 510 nm for emission. Measuring time was 20 ms. After the fluorescence measurement 100 μl of luciferin in 0.1 M citric acid-sodium citrate buffer (pH 5.0) was dispensed into the wells and the plate was shaken

for two minutes (shaking diameter 1 mm, 1 020 rpm), after which luminescence was recorded with a measuring time of 1000 ms.

Plating

[0035] Samples for plating were diluted 10^2 to 10^7 fold with 150 mM NaCl and plated onto L agar plates (L broth containing 1.6 % agar). Colonies were counted after overnight incubation at 37 °C.

Live/dead staining and Flow cytometric analysis

[0036] Bacteria from 1 000 μl of cell culture were used for live/dead staining and flow cytometric analysis using a LIVE/DEAD BacLight bacterial viability kit (catalogue no. L-7005) for microscopy and quantitative analysis obtained from Molecular Probes Europe (Leiden, The Netherlands) and Fluoresbrite beads (diameter, 1.8 μm) obtained from Polysciences Inc. (Warrington, Pa.) as described in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.

Results

[0037] When the cultures were transferred to +30 °C, the cells grew logarithmically for 1-4 hours depending on the initial cell concentration. Luminescence and fluorescence rose logarithmically and were essentially constant per cell. Thus cell number could be assessed based on luminescence or fluorescence.

[0038] When ethanol was added in different concentrations to the growth medium (see Figures 4 and 5) death was, after a very short incubation period of 5 min, more or less unsignificant at ethanol concentrations below 5 % and became more significant with increasing ethanol concentration reaching very pronounced significance at ethanol concentrations above 10 %. Correspondingly fluorescence (Figure 2) was essentially constant whatever the ethanol concentration in spite of dramatically decreasing corresponding live cell count according to plate count (Figure 4) and percentage of live cells according to the live/dead staining (Figure 5) whereas luminescence (Figure 3) dropped dramatically essentially corresponding to the dramatic drop in plate count (Figure 4) and the percentage of live cells (Figure 5) with increased ethanol concentration.

[0039] The effect of serum complement on the growth and death of *E. coli* is shown in Figures 6 to 8. Fluorescence (Figure 6) and luminescence (Figure 7) are shown before (squares) and after (circles) incubation for 90 minutes with serum complement. Fluorescence (Figure 6) is

slightly increased, during incubation regardless of the concentration of serum, whereas luminescence (Figure 7) decreases during incubation with increasing serum concentration. The decrease of luminescence during incubation with increasing concentrations of serum correlates clearly with the percentage of cells alive after incubation (Figure 8).

CLAIMS

1. A method to enable the assessment of the growth rate and death rate of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism at least two reporter genes, which method is characterised in that

a) said reporter genes code for luminescent and/or fluorescent products and within said time period and environment at least two said products of the following are produced:

i) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period,

ii) a product present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period, and

iii) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that have died within said chosen time period,

and said products can be measured through their luminescence and/or fluorescence;

b) said micro-organism is incubated within the environment of interest and said luminescence and/or fluorescence is detected after said chosen time period, and

c) the growth and death rate of the said micro-organism is assessed based on at least two of the following:

i) the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period,

ii) the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period, and

iii) the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

2. The method according to claim 1 characterised in that said micro-organism is a gram negative bacteria, e.g. *Escherichia coli*.

3. The method according to claim 1 characterised in that

a) one reporter gene coding for a luminescent product is luciferase, which is used for the determination of amount of cells alive at any moment within said chosen time period, and

b) another reporter gene coding for a fluorescent product is green fluorescent protein (GFP), which is used for the determination of total amount of cells of said micro-organism that are or have been alive within said chosen time period.

4. The method according to claim 1 characterised in that said reporter genes are introduced into said micro-organism in a plasmid.

5. The method according to claim 3 characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

6. The method according to claim 2 characterised in that

a) one reporter gene coding for a luminescent product is luciferase, which is used for the determination of amount of cells alive at any moment within said chosen time period, and

b) another reporter gene coding for a fluorescent product is green fluorescent protein (GFP), which is used for the determination of total amount of cells of said micro-organism that are or have been alive within said chosen time period.

7. The method according to claim 2 characterised in that said reporter genes are introduced into said micro-organism in a plasmid.

8. The method according to claim 4 characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

9. The method according to claim 6 characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

10. The method according to claim 7 characterised in that said plasmid is pGFP+luc* (SEQ ID NO: 1).

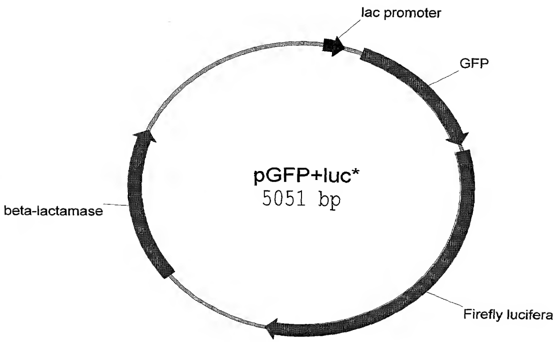
ABSTRACT

[0040] A method to enable the assessment of the growth rate and death rate of a micro-organism within a chosen time period in an environment of interest. The method is characterised in that

a) two reporter genes are introduced to said micro-organism wherein, the reporter genes used code for luminescent and/or fluorescent products and at least two of the following products: an essentially stable product produced in an essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period; a product present in an essentially known proportion to the amount of cells alive at any moment within said chosen time period; and an essentially stable product produced in an essentially known proportion to the total amount of cells of the said micro-organism that have died within said chosen time period, and said products can be measured through their luminescence and/or fluorescence;

b) the said micro-organism is incubated and said luminescence and/or fluorescence is detected after said chosen time periods, and

c) the growth and death rate of the said micro-organism is assessed based on at least two of the following: the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period; the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period; and the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.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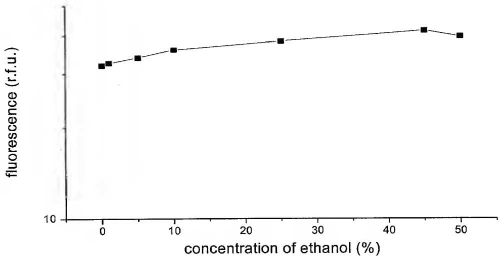
Firefly lucifera

Figure 1



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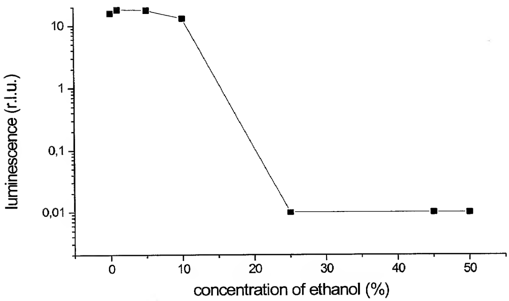

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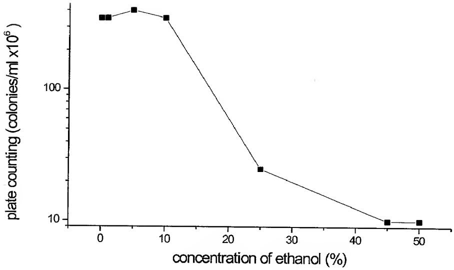

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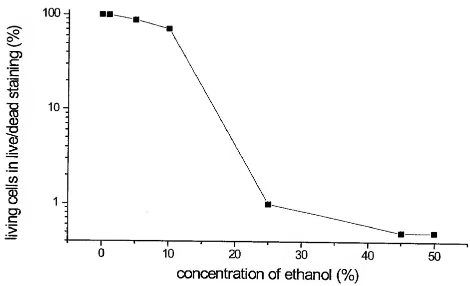
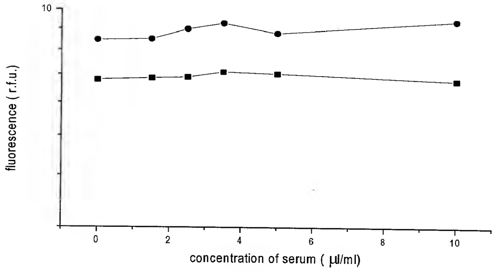


Figure 5

09/980585



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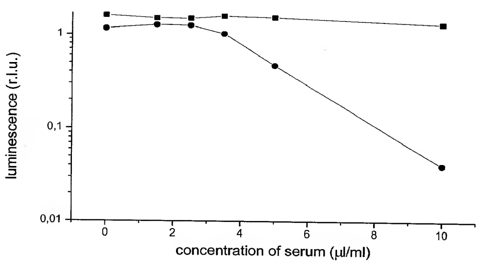

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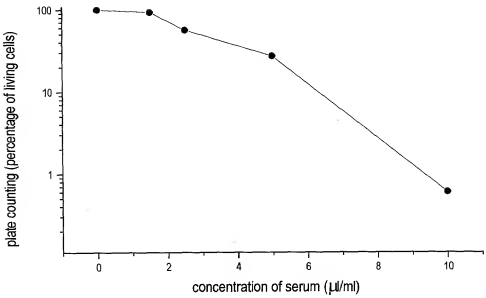
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100
10
1
0
2
4
6
8
10
concentration of serum (µl/ml)

Figure 8

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)	Attorney Docket No.	
___ Declaration Submitted with Initial Filing ___ Declaration Submitted after Initial Filing	First Named Inventor	
	COMPLETE IF KNOWN	
	Application Number	
	Filing Date	
	Group Art Unit	
	Examiner Name	

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: A method to enable assessment of growth and death of micro-organisms

___ the specification of which is attached hereto.

X the specification of which was filed on December 4, 2001 as United States Application Number 09/980,585 or PCT International Application Number PCT/FI00/00507 and was amended on December 4, 2001

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
991296	FI	06/07/1999	___	___	X
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I or we hereby appoint the registered practitioner(s) associated with Customer No. **6449** to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Direct all correspondence to Customer Number **6449**.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
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Mailing Address Vaakunatie 10			
Mailing Address			
City Kaarina	State	Zip FIN-20780	Country Finland

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Inventor's Signature		Date 21.1.2002	
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Mailing Address			
City Turku	State	Zip FIN-20100	Country Finland

NAME OF THIRD INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
Given Name (first and middle [if any])		Family Name or Surname	
Inventor's Signature		Date	
Residence: City	State	Country	Citizenship
Mailing Address			
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VIRTA, Marko

<120> A Method to Enable Assessment of Growth and Death of Micro-organisms

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Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
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Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
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Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
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Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
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Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
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Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
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Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
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Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
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Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
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Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
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Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
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Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
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Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
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Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
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Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
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Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
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35 40 45

Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe
50 55 60

Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser
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Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser
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Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
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Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser
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Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser
225                 230                 235                 240

Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile
                245                 250                 255

Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn
            260                 265                 270

Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp
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— There are only 5 Drawing not 8.